Apoptosis Kit

FlowTACS™ Apoptosis Detection Kit

Catalog Number: 4817-60-K

Detection of apoptosis using flow cytometry for 60 samples.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

SECTION	PAGE
BACKGROUND	1
LIMITATIONS OF THE PROCEDURE	1
TECHNICAL HINTS	1
PRECAUTIONS	1
MATERIALS PROVIDED & STORAGE CONDITIONS	2
OTHER MATERIALS REQUIRED	2
REAGENT PREPARATION	3
ASSAY PROTOCOL	4
CONTROLS	5
DATA INTERPRETATION	6
TROUBLESHOOTING	7
APPENDICES	8
REFERENCES	10

Manufactured and Distributed by:

USA R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413
TEL: 800 343 7475   612 379 2956
FAX: 612 656 4400
E-MAIL: info@bio-techne.com

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane, Abingdon Science Park
Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China Bio-Techne China Co., Ltd.
Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373   (400) 821-3475
FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

BACKGROUND

Apoptosis is characterized by a number of intracellular phenomena such as membrane blebbing, chromatin condensation and nuclear DNA fragmentation. This DNA fragmentation provides the basis for several assays used to detect apoptosis *in situ*. During apoptosis, DNA cleavage occurs, most typically within the linker regions of DNA between nucleosomes. Extraction of DNA from apoptotic cells and analysis by agarose gel electrophoresis reveals a ladder pattern representing multiples of approximately 185 bp. This cleavage generates free 3'-hydroxyl residues that can be utilized by terminal deoxynucleotidyl transferase (TdT) in end-labeling reactions. Incorporation of biotinylated nucleotides into the DNA by TdT allows detection with streptavidin-linked conjugates.

The FlowTACS Apoptosis Detection Kit provides enough reagents to supply 60 samples and is supplied with a Strep-Fluorescein conjugate for detection of DNA fragmentation providing a fluorescent readout. The FlowTACS Apoptosis Detection Kit can be used for the detection of apoptosis using flow cytometry, direct visualization using a fluorescent microscope, or for quantitation using a fluorometer. The protocol given here describes the use of the kit in flow cytometry and hints and tips for double labeling are provided in the Appendix.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between sample additions and reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTIONS

The acute and chronic effects of overexposure to reagents of this kit are unknown. Safe laboratory procedures should be followed, and protective clothing should be worn when handling kit reagents.

The FlowTACS™ Apoptosis Detection Kit contains reagents that are harmful if swallowed or in contact with skin, and irritating to the eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
TdT dNTP Mix	4810-30-04	35 µL	Store at ≤ -20 °C.
TdT Enzyme	4810-30-05	30 µL	
50X Mn^{2+}	4810-30-14	50 µL	
TACS-Nuclease™	4800-30-15	15 µL	
Strep-Fluorescein	4800-30-14	30 µL	Store at ≤ -20 °C **in the dark.**
Propidium Iodide/RNAse	4817-60-04	1 mL	Store at 2-8 °C **in the dark.**
Cytonin™	4876-05-01	6 mL	Store at 2-8 °C.
TACS-Nuclease Buffer	4800-30-16	1.5 mL	
10X TdT Labeling Buffer	4810-60-02	20 mL	
10X TdT Stop Buffer	4810-60-03	20 mL	

OTHER MATERIALS REQUIRED

Equipment:

- Pipette and pipette tips
- 37 °C incubator
- 50 and 500 mL graduated cylinders
- ≤ -20 °C and 2-8 °C storage
- Ice bucket
- Fluorescence microscope
- Flow cytometer
- Timer

Reagents:

- 10X Phosphate Buffered Saline (PBS)
- 37% Formaldehyde
- Distilled water

Disposables:

- Treated Glass Microscope Slides (or alternative support)
- 50 mL tubes
- 1-200 µL and 200-1000 µL pipette tips
- Microcentrifuge tubes
- Ice
- 1.5 and 10 mL serological pipettes
- Gloves
- Flow cytometry tubes
- Aluminum foil

REAGENT PREPARATION

Reagents marked with an asterisk (*) should be prepared immediately before use.

1X PBS - Approximately 500 mL of 1X PBS is used to process 20 samples. Dilute 10X PBS to 1X using distilled water. Store 1X PBS at room temperature.

***3.7% Buffered Formaldehyde** - If required, 20 mL of freshly prepared fixative is used to process 20 samples. To prepare add:

Reaction Component	Volume
37% Formaldehyde	2 mL
10X PBS	2 mL
Distilled water	16 mL

Wear gloves and exercise caution when handling formaldehyde solutions.

Cytonin - Provided ready to use. If required, 100 µL of Cytonin is used per sample. Discard if solution is cloudy.

1X TdT Labeling Buffer - Dilute the 10X TdT Labeling Buffer to 1X using distilled water. Leave at room temperature until use. Remove an aliquot of 25 µL per sample for preparing the Labeling Reaction Mix (see below) and place on ice.

***Labeling Reaction Mix** - Thaw 50X Mn^{2+} and TdT dNTP Mix at room temperature, then place on ice. To maintain optimal enzyme activity, remove the TdT Enzyme tube from freezer only long enough to pipette the required volume. Alternatively, place the TdT Enzyme in a ≤ -20 °C freezer block. Prepare the Labeling Reaction Mix just before use and keep the prepared reaction mix on ice. Prepare 50 µL per sample in the sequence given below:

Reaction Component	2 Samples	10 Samples	n Samples
TdT dNTP Mix	1 µL	5 µL	n x 0.5 µL
TdT Enzyme	1 µL	5 µL	n x 0.5 µL
50X Mn^{2+}	1 µL	5 µL	n x 0.5 µL
1X TdT Labeling Buffer	50 µL	250 µL	n x 25 µL

1X TdT Stop Buffer - Dilute the 10X TdT Stop Buffer to 1X using distilled water. Leave at room temperature until use. Use 1 mL of 1X TdT Stop Buffer to process 1-10 samples.

***Strep-Fluorescein** - Use 25 µL of Strep-Fluorescein Solution per sample under dimmed lights. Store prepared Strep-Fluorescein on ice in the dark until use. To prepare add:

Reaction Component	1-4 Samples	10 Samples	n Samples
1X PBS	200 µL	500 µL	n x 50 µL
Strep-Fluorescein	1 µL	2.5 µL	n x 0.25 µL

For research use only. Not for use in diagnostic procedures.

REAGENT PREPARATION *Continued*

Propidium Iodide/RNAse Solution - The Propidium Iodide/RNAse solution can be added directly to the sample just prior to flow cytometry. Typically 10 µL per 500 µL sample is sufficient. Incubate at room temperature for 5 minutes then analyze.

***TACS-Nuclease Solution** - For the preparation of a Nuclease-treated positive control sample, it is recommended that the DNA breaks be generated in a separate step in this case, TACS-Nuclease should be diluted 1:25 in TACS Nuclease Buffer, as below:

Reaction Component	per control
TACS-Nuclease Buffer	25 µL
TACS-Nuclease	1 µL

ASSAY PROTOCOL

It is important to read through the instructions before preparing tissue or cell samples for labeling. There are key steps that are very important for successful labeling.

1. Pellet 1 x 10^6 cells at 1000 x g for 5 minutes at room temperature. Draw off medium with a Pasteur pipette. A small residual volume of medium may be left.
2. Resuspend cells gently in 1 mL of 3.7% Buffered Formaldehyde. Leave to stand at room temperature for 10 minutes. It is not necessary to wash cells prior to fixation.
   Note: *See Appendix for alternative fixation methods and storage of fixed cells.*
3. Pellet fixed cells at 1500 x g for 5 minutes at room temperature. Draw off fixative with a Pasteur pipette. Do not disturb cell pellet. A small residual volume of fixative may be left (~50 µL).
4. Add 100 µL of Cytonin and tap tube several times to resuspend cells. Leave at room temperature for 30 minutes. Tap tube occasionally to keep cells in suspension. Samples can be stored in Cytonin overnight at 2-8 °C.
5. Prepare the 1X Labeling Buffers, labeling mixes and 1X Stop Buffer for the next steps. Refer to Reagent Preparation.
6. Pellet cells at 1500 x g for 5 minutes at room temperature. Draw off Cytonin with Pasteur pipette. Do not disturb cell pellet. A small residual volume of Cytonin may be left (~50 µL).
7. Wash cells by resuspending in 1 mL of 1X TdT Labeling Buffer then repeating the centrifugation at 1500 x g for 5 minutes at room temperature. Draw off excess buffer with a Pasteur pipette.
   Note: *Nuclease treat one sample after Cytonin treatment prior to step 6. The other samples can remain in 1X TdT Labeling Buffer for the additional 30 minutes.*

8. Add 25 µL of Labeling Reaction Mix and resuspend by gently tapping tube. Incubate in 37 °C water bath for 1 hour. Tap tube gently every 10 minutes to keep cells in suspension. Shorter incubation times (10-30 minutes) may be applicable for some samples. Remember to include the "no enzyme" and TACS-Nuclease-treated Control.
9. Add 1 mL of 1X TdT Stop Buffer, tap tube to mix. Pellet at 1500 x g for 5 minutes at room temperature. Draw off 1X TdT Stop Buffer with Pasteur pipette.
10. Add 25 µL of the diluted Strep-Fluorescein and incubate at room temperature in the dark for 10 minutes. Other streptavidin-fluorochrome conjugates may be substituted.
11. Pellet cells at 1500 x g for 5 minutes at room temperature. Remove the Strep-Fluorescein with a Pasteur pipette and resuspend cells in 500 µL of 1X PBS. Additional washes may be required if there is high background fluorescence in the controls.
12. Add 10 µL of Propidium Iodide/RNAse solution, if required, incubate at room temperature for 5 minutes. Place samples on ice in the dark until assayed. Perform flow cytometry within 2 hours.

CONTROLS

The minimum number of controls that should be included when running your assay are:

TACS Nuclease-treated Control. Treat one of your samples with TACS-Nuclease to generate DNA breaks in every cell. This sample will confirm that the labeling reaction has worked and can be used to determine the initial settings for flow cytometry.

1. Wash cells in 1X PBS then resuspend in 25 µL of TACS Nuclease Solution.
2. Incubate at room temperature for 30 minutes.
3. Add 1 mL of 1X PBS.
4. Pellet cells at 1500 x g for 5 minutes at room temperature.
5. Discard TACS-Nuclease Solution and continue from Step 6 in the Assay Protocol.

Unlabeled Experimental Sample. The TdT Enzyme should be omitted from the labeling reaction mix. This control will indicate the level of background fluorescence associated with non-specific binding of the Strep-Fluorescein or if increased washes may be needed. This sample can be used to determine the settings for flow cytometry.

Control Cells. An appropriate experimental control should be included in each experiment.

Propidium Iodide/RNAse Solution. If propidium iodide is used perform each of the above controls in duplicate so that the samples can be assayed with and without propidium iodide.

DATA INTERPRETATION

Apoptosis is often defined by morphological criteria. It is important to consider the data obtained from standard microscopy and histochemistry in conjunction with biochemical assays used for confirmation of apoptosis.

Using Strep-Fluorescein Label only

Apoptosis is indicated by positive fluorescence an order of magnitude greater than the unlabeled control sample (TdT Enzyme omitted from Labeling Reaction Mix). There may be some spread in the degree of fluorescence of positive cells due to variability in the number of DNA breaks per cell. Different cell types may exhibit different degrees of fluorescence, therefore comparison with an unlabeled control cell of the same type under study is important for interpretation of data. The TACS Nuclease-treated Control sample confirms satisfactory labeling.
Note: *The nuclease-treated cells will not necessarily be condensed and a slightly different profile on flow cytometry is to be expected.*

Using Strep-Fluorescein and Propidium Iodide

All cells are labeled by Propidium Iodide, after fixation and permeabilization, allowing the entire cell population to be visualized by flow cytometry. Propidium Iodide fluorescence can also be used to aid in distinguishing between apoptotic (typically condensed and fragmented nuclei) and necrotic (swollen nuclei) cells. Propidium Iodide may allow distinction to be made between these two modes of cell death when used in conjunction with Strep-Fluorescein fluorescence and analysis of size by flow cytometry. The labeling by Strep-Fluorescein should not be affected by the inclusion of Propidium Iodide if the flow cytometry apparatus settings are appropriately compensated. Typically the Strep-Fluorescein is recorded on the FL1 channel and the Propidium Iodide on the FL2 channel. Use a TACS-Nuclease-treated Control or a positive (*i.e.* a known apoptotic) experimental sample without Propidium Iodide to set the FL1 compensation and an unlabeled (enzyme omitted) experimental sample with Propidium Iodide to set the FL2 compensation.

TROUBLESHOOTING

Rule out major problems by checking the labeling in the control samples first.

Problem	Cause	Action
No Strep-Fluorescein labeling of positive control (TACS-Nuclease-treated or known apoptotic sample).	Enzyme inactive (most labile component).	Enzyme must be stored at <-20 °C in manual defrost freezer. Do not bring enzyme up to ice temperature. Place in <-20 °C freezer block or remove aliquot from tube directly at freezer.
	Inadequate TACS-Nuclease treatment.	Increase incubation time.
	Strep-Fluorescein was frozen.	Ensure that on arrival the Strep-Fluorescein was placed at 2-8 °C for storage in the dark.
	Inadequate permeabilization or over fixation.	Time of Cytonin treatment can be increased up to 24 hours (perform at 2-8 °C for incubations of 2 hours or more). Avoid extensive fixation in strong cross-linking agents.
Labeling of majority of cells in negative control (*i.e.* TdT Enzyme omitted from Labeling Reaction Mix).	Non-specific binding of Strep-Fluorescein.	Add in an additional wash with PBS before and after labeling with Strep-Fluorescein. Include 1% BSA in the Strep-Fluorescein. Dilute the Strep-Fluorescein to 1:500 instead of 1:200 in PBS.
All cells exhibit low level fluorescence.	Autofluorescence	Gate flow cytometry channels appropriately to compensate. Avoid paraformaldehyde fixation.
	Cells damaged during procedure.	Avoid aggressive resuspension and perform only low speed centrifugations.
No labeling in experimental sample (positive controls label).	No apoptosis (or necrosis) occurring in sample.	Check morphology of cells prior to assay for characteristics of apoptosis.
Extensive labeling in experimental control (*i.e.* healthy cells).	High rate of cell death (apoptosis or necrosis).	Check culture conditions. Reduce time of assay.
Overlap of relative fluorescence of Strep-Fluorescein between controls and experimental sample	Incorrect or inadequate compensation settings/ gates.	Use the appropriate controls to set gates and compensation on FL1 and FL2 channels.

APPENDICES

Appendix A. Fixation Methods

There are several common fixation methods that are appropriate for use with the protocol described here. Formaldehyde is the recommended fixative based on laboratory testing and data obtained from our laboratory start-up service. However, other fixatives that maintain DNA integrity may be used also. These include alcohol fixatives such as ethanol, methanol or acetone. Paraformaldehyde is not recommended due to autofluorescence of paraformaldehyde-fixed samples. Glutaraldehyde has poor penetration but remains as an alternative to formaldehyde. Fixation method will likely be dictated by immunocytochemistry protocols in double labeling experiments.

If you wish to store fixed cells, it is recommended to fix formaldehyde followed by a 20 minute postfix in 100% methanol. Wash in PBS then store in Cytonin at 2-8 °C for up to 1 month. Proceed with a wash in 1X Labeling Buffer then continue with the labeling reaction. Note that if cells are fixed using alcohol *e.g.* ethanol, there will be leakage of small DNA fragments from apoptotic cells and the propidium iodide profile will be different compared to cells labeled without prolonged storage times. The cross-linking fixatives such as formaldehyde limit DNA loss.

Appendix B. Double Labeling Hints and Tips

The *in situ* labeling protocol described here is useful for double labeling experiments when the occurrence of apoptosis can be correlated with cellular antigens against which antibodies are available. The rapid assay for apoptosis detection using Annexin V-FITC or Annexin V-Biotin uses unfixed and unpermeabilized cells and is therefore only applicable for double labeling when native cell surface markers are being detected along with DNA fragmentation.

The key to double labeling experiments is determining fixation and permeabilization conditions under which both antigen and DNA integrity are maintained. Post-treatments used in immunocytochemistry to permeabilize or expose antigenic determinants include treatment with proteases, acid or base, detergent and microwaving. Permeabilization with Cytonin may be sufficient for many antibodies and additional treatment may not be needed. Protease treatment may be performed but should be kept to a minimum to avoid complete cell destruction. Acid or base treatment should be avoided. Microwaving is an option that has given excellent results in double labeling experiments but requires careful experimentation to determine the correct wattage, time and cooling cycles for each experimental sample (Shi. *et al.*, 1997).

It is recommended that conditions for DNA labeling and immunocytochemistry are optimized in separate experiments using conditions of fixation and permeabilization that will be appropriate for both methods. Once each procedure has been successful independently they can be combined as follows:

For research use only. Not for use in diagnostic procedures.

Method

1. Fix and permeabilize (maintain DNA and antigen integrity).
2. Bind primary antibody, wash.
3. Perform DNA labeling procedure (start with wash in 1X Labeling Buffer) up to wash, with 1X TdT Stop Buffer prior to labeling with Strep-Fluorescein.
4. Combine Strep-Fluorescein and secondary tagged antibody (fluorochrome other than FITC) and incubate with cells for 30 minutes.
5. Wash with PBS and perform flow cytometry.

Times and temperature of incubations should be optimized for the system under study. In tricolor experiments (*e.g.* propidium iodide and FITC for FlowTACS plus fluorochrome for antigen identification) select a chromophore that is compatible with FITC and propidium iodide and with the filters you have available on the flow cytometry equipment. Phycoerythrin in combination with propidium iodide and FITC provides good contrast.

Remember to carefully plan and include controls in addition to the ones recommended for detection of apoptosis to allow interpretation of double-labeled samples.

Appendix C. Preparation of Adherent Cells.

Detection of apoptosis using flow cytometric analysis of a fluorescent tag is most convenient for suspension cells. Adherent cells require lifting from the substrate on which they are growing prior to fixation and permeabilization. Adherent cells can be treated with trypsin or pepsin. The inclusion of EDTA will improve the efficiency of trypsinization. Most cells can be trypsinized in about 5 minutes at 37 °C in 0.5% Trypsin, 2 mM EDTA. The trypsinization should be stopped by the addition of complete medium (containing fetal bovine serum) followed by two washes in 1X PBS. Use of Proteinase K or Trypsin may destroy certain antigens and should therefore not be used if immunocytochemistry is also to be performed, unless antigen retrieval using proteases is recommended for the antigen of interest.

As an alternative to proteases, treat the cells with 2 mM EDTA in PBS for 5 minutes at 37 °C then scrape the cells from the culture vessel using a rubber policeman. Do not treat cells for extensive periods of time with EDTA because the chelation of calcium by EDTA may alter cell morphology etc. prior to fixation. Cells may also be scraped directly without EDTA treatment but this method tends to generate clumps of cells that are not easily dispersed into single cell suspension and increases the amount of cell debris.

REFERENCES

1. Ormerod, M.G. (1994) Flow Cytometry: a Practical Approach. 2nd Edition. IRL Oxford University Press, Inc.
2. Negoescu, A. *et al*. (1996) J. Histochem. Cytochem. **44**:959.
3. Shi, S.-R. *et al*. (1997) J. Histotechnology **20**:145.

All trademarks and registered trademarks are the property of their respective owners.

©2020 R&D Systems®, Inc.

06.20 3094817.1 6/20